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WPI Abstract Accession No. 92-131866/16 & WO 92/04891 A1

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INT CL^6 **A61K 9/16 9/50 38/18**
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(54) **Sustained release of erythropoietin from microspheres**

(57) Polylactide-co-glycolide (PLG) microspheres containing erythropoietin (EPO) are produced by an oil-in-water emulsion process and have a bimodal size distribution which enables sustained release of EPO. The microspheres may be injected parenterally or subcutaneously.

GB 2 316 316 A

Figure 1

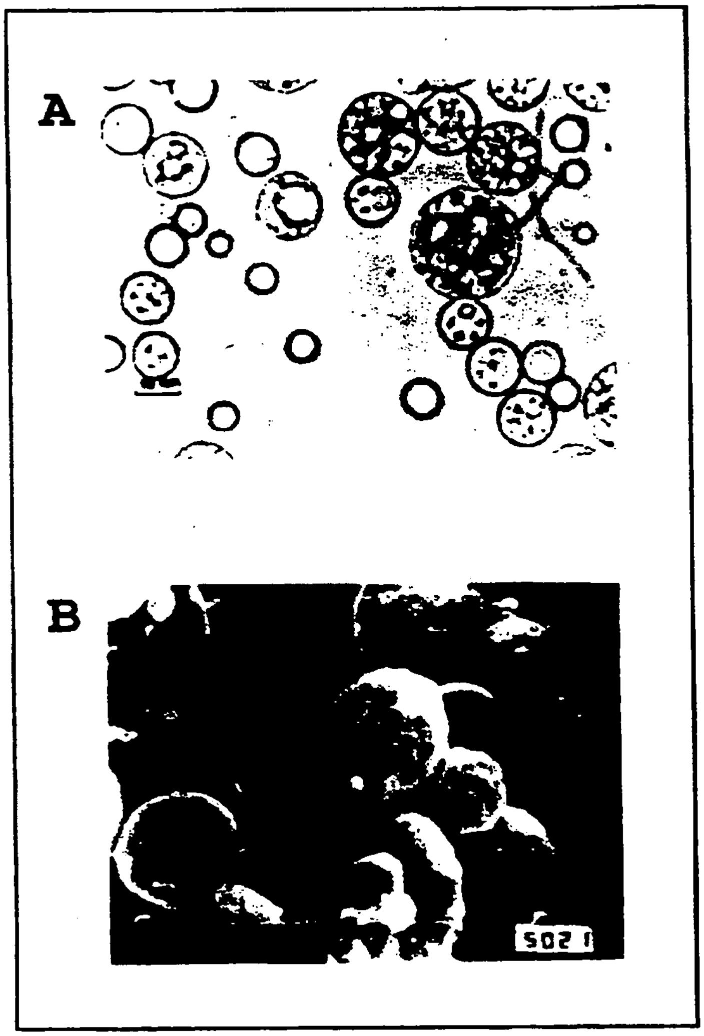
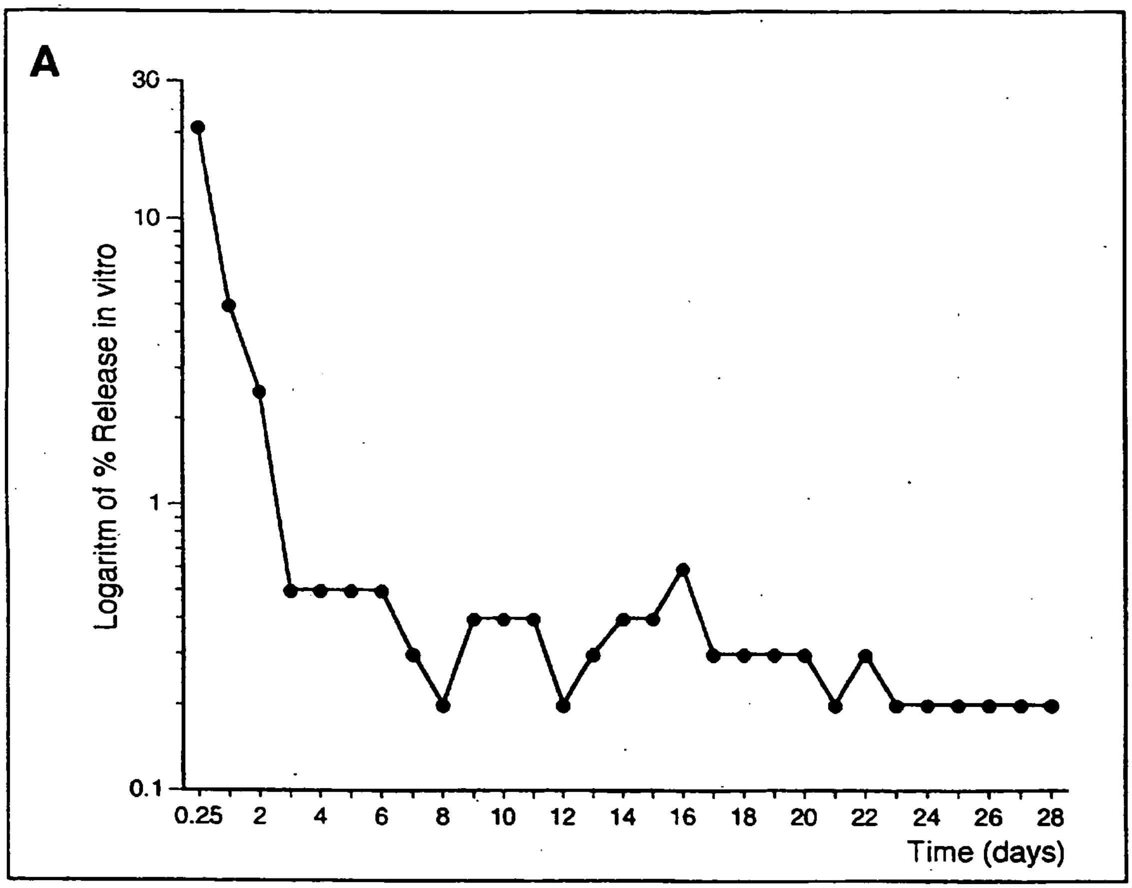


Figure 2


A
30
10
1
0.1
Logaritm of % Release in vitro
0.25
2
4
6
8
10
12
14
16
18
20
22
24
26
28
Time (days)

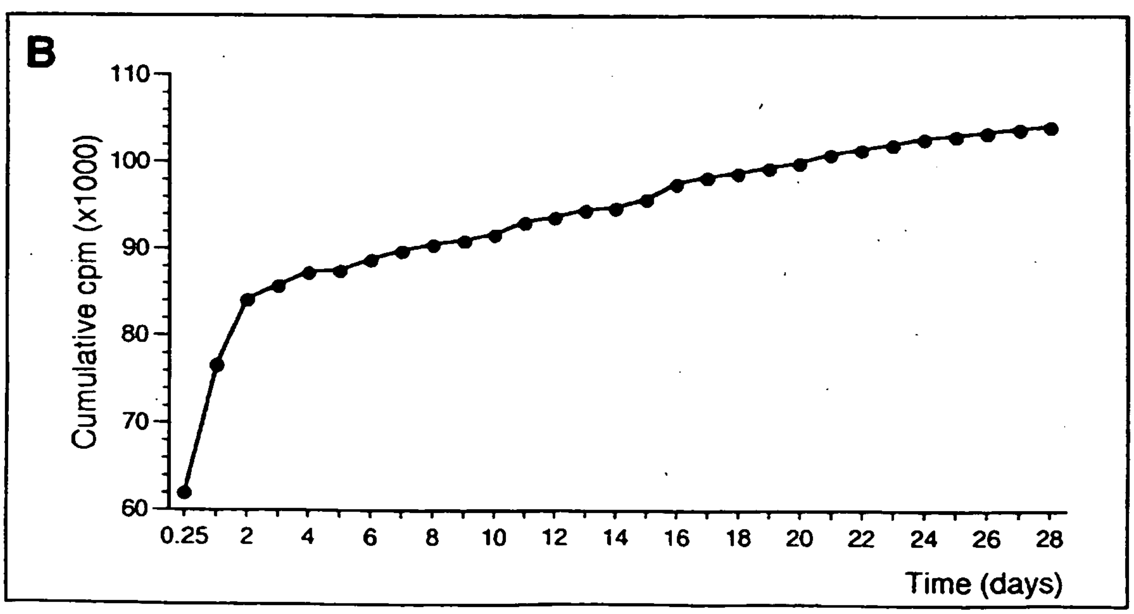

B
110
100
90
80
70
60
Cumulative cpm (x1000)
0.25
2
4
6
8
10
12
14
16
18
20
22
24
26
28
Time (days)

Figure 3

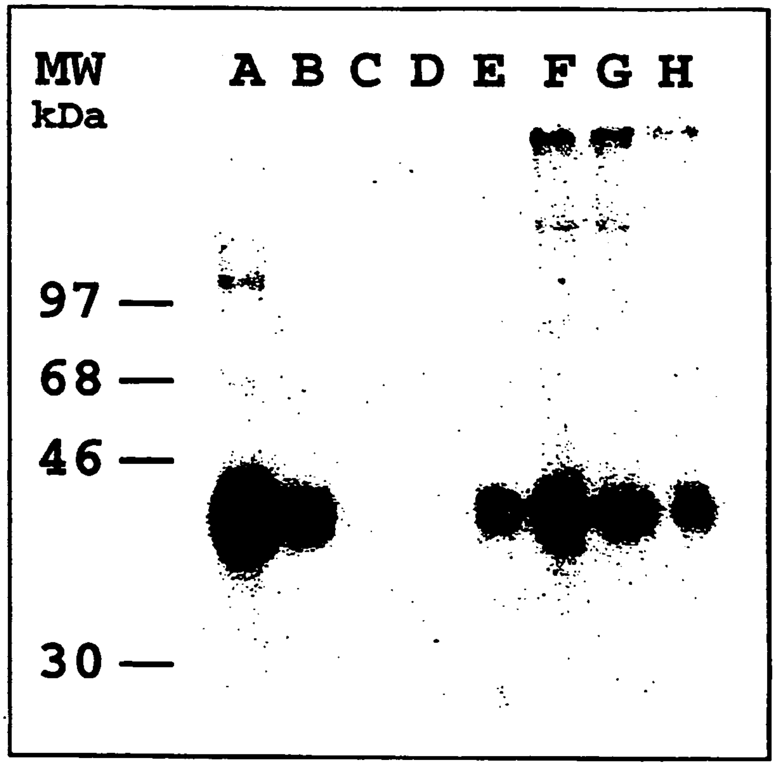
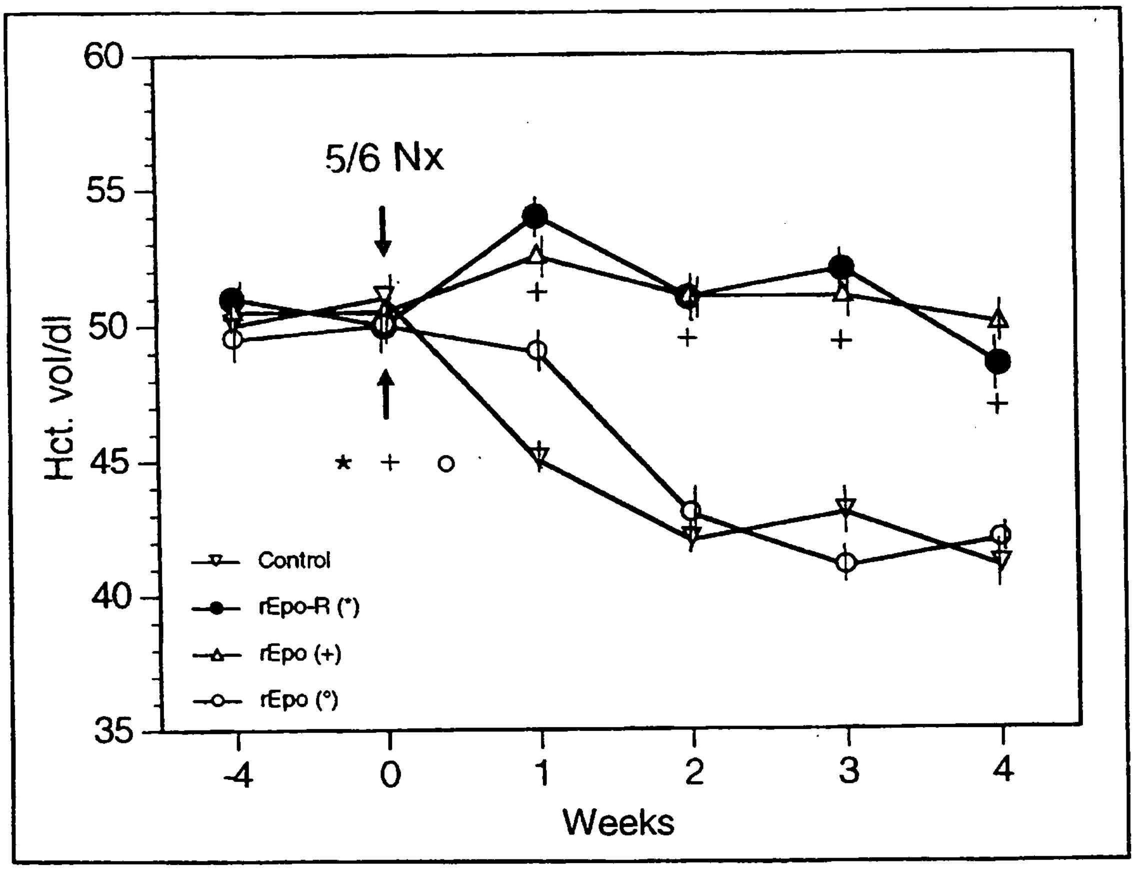


Figure 4



5/6 Nx
Hct. vol/dl
Weeks
60
55
50
45
40
35
-4
0
1
2
3
4
Control
rEpo-R (*)
rEpo (+)
rEpo (°)

Figure 5

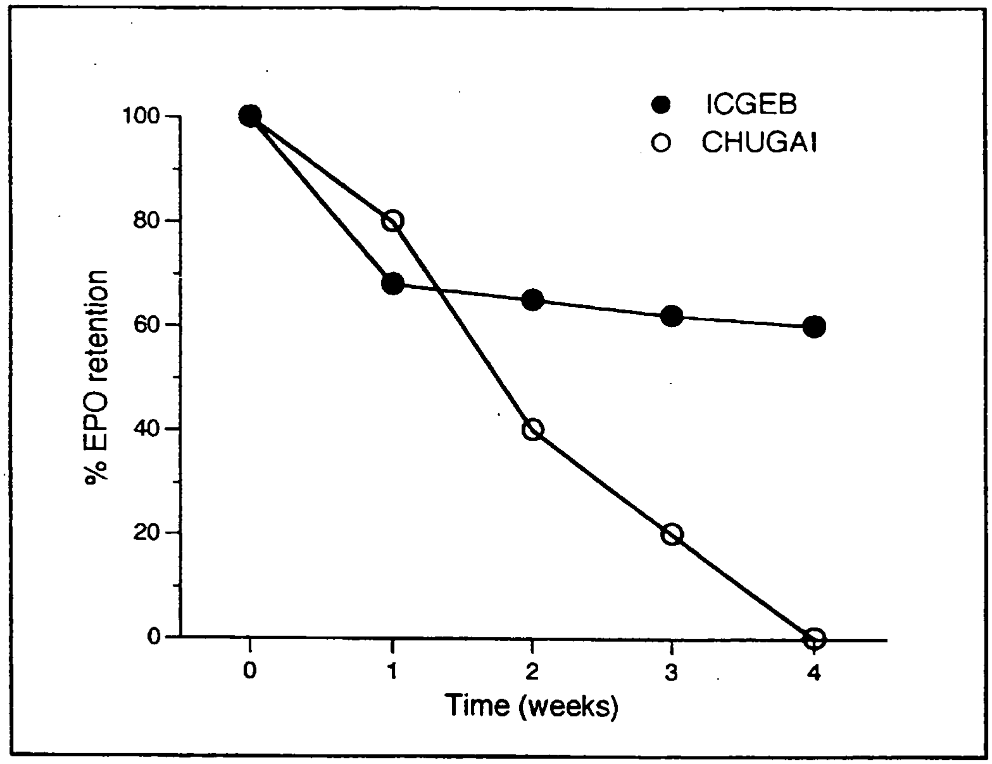
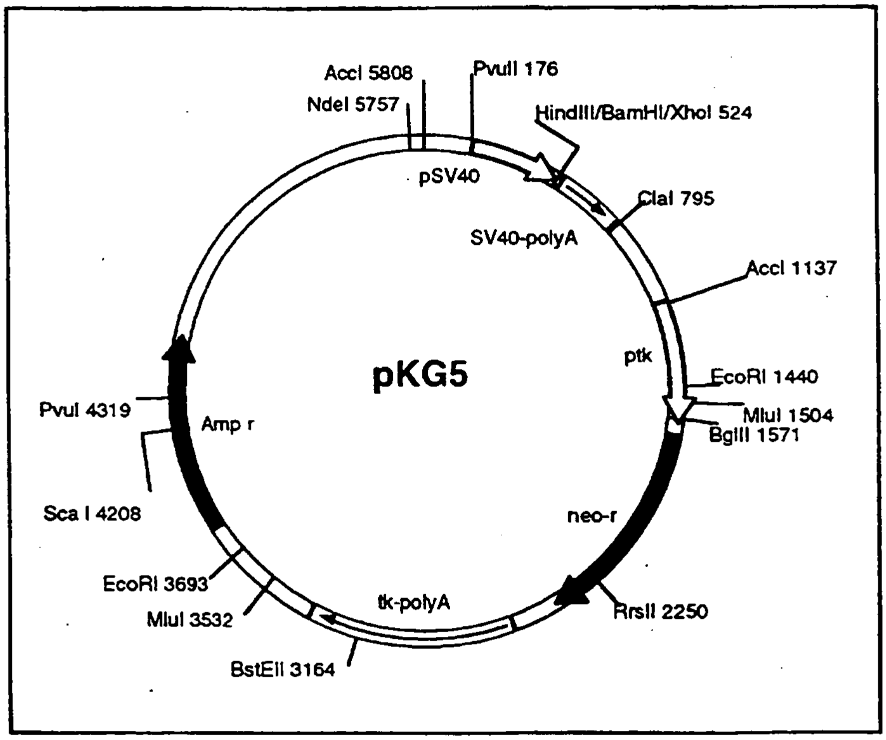
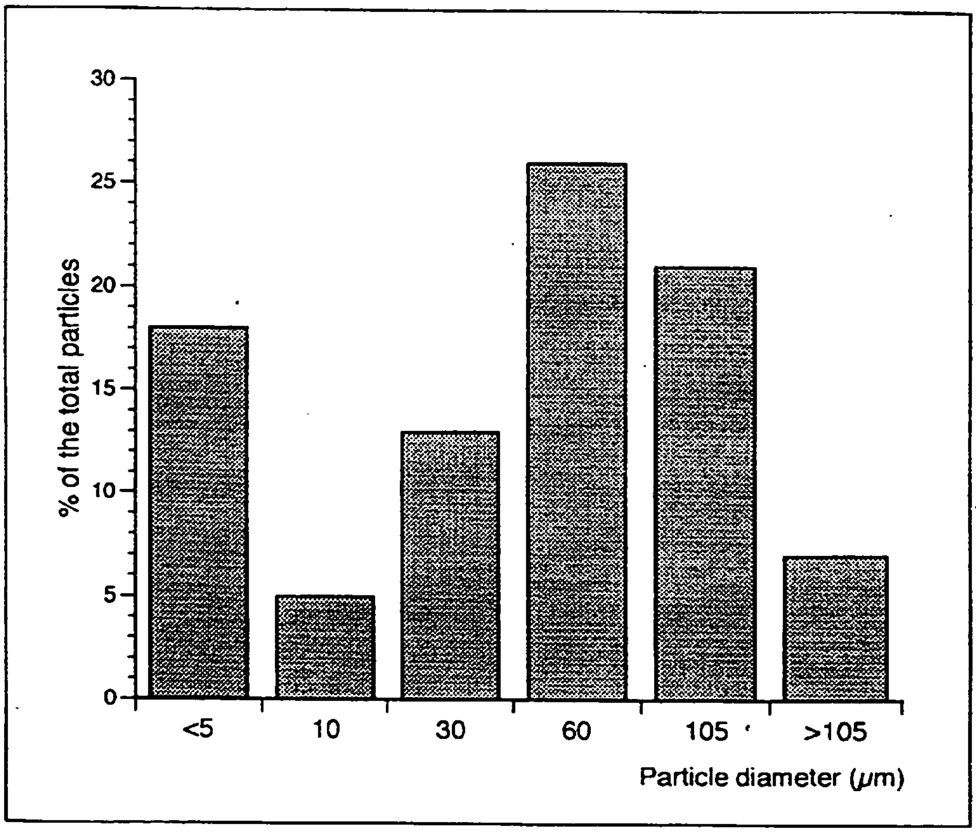


Figure 6



pKG5
AccI 5808
NdeI 5757
PvuII 176
HindIII/BamHI/XhoI 524
pSV40
ClaI 795
SV40-polyA
AccI 1137
ptk
EcoRI 1440
MluI 1504
BglII 1571
neo-r
RsrII 2250
tk-polyA
BstEII 3164
MluI 3532
EcoRI 3693
Sca I 4208
PvuI 4319
Amp r

Figure 7



30
25
20
15
10
5
0
% of the total particles
<5
10
30
60
105
>105
Particle diameter (μm)

2316316

SUSTAINED-RELEASE PREPARATION

The present invention relates to a sustained-release preparation for the delivery of erythropoietin (EPO) to mammalian subjects, typically humans.

EPO stimulates the proliferation and differentiation of erythroid cells, leading to an increase in the number of reticulocytes in the blood, which consequently increases the blood's haematocrit and haemoglobin concentration.

Erythropoietin (EPO) is a sialo-glycoprotein of molecular weight 34,000 Daltons, of which about 40% is accounted for by glycosidic chains. EPO was purified for the first time in sufficient amounts for biochemical studies in 1977. In 1984 its cDNA was isolated and EPO was produced in bacterial cells. This material, although biologically active *in vitro*, was not efficient *in vivo* because the lack of postranslational modification (mainly glycosylation) dramatically shortens EPO's half-life *in vivo*. EPO produced in baculovirus/insect cell systems is also available. This EPO is glycosylated according to the scheme characteristic of the insect cells and, like bacterial EPO, has a shortened half life *in vivo* owing to the lack of mammalian-style glycosylation. Hence, neither bacterial EPO nor baculovirus-derived EPO is suitable for therapeutic use in isolation.

The expression of mature glycosylated EPO in mammalian Chinese Hamster Ovary (CHO) and BHK cells and improved purification procedures eventually yielded a satisfactory EPO for use *in vivo*. Nonetheless, even this fully glycosylated EPO has some serious drawbacks when administered in isolation.

If large quantities (greater than about 150 units of EPO per kg of body mass) are administered to patients, a number of side-effects are experienced. In particular, there is a risk of thrombosis and increased blood pressure, which may require concomitant treatment to be carried out for hypertension.

These risks can be alleviated somewhat if the EPO is administered gradually to take the haematocrit to 30-32%; the more gradually the haematocrit is increased, the less the risk of hypertension.

Current therapeutic schemes of this type nonetheless involve up to 3 weekly injections of 50 to 150 U/Kg of EPO. The therapy aims to increase the haematocrit by 1 to 2 points per week.

For long term maintenance therapy, the dosage can be reduced but both initial therapy and maintenance involve repeated injections that produce peaks of high EPO concentration in the bloodstream followed by a rapid decrease and eventual disappearance from circulation after 5-10 hours of the injection.

Therefore, owing to the short half-life of EPO, especially bacterially-produced and baculovirus-produced EPO, all methods of therapy that rely on the administration of EPO in isolation are inherently limited as they necessitate repeated injections. These are inconvenient, and generate undesirable peaks in EPO concentration. Further, as EPO levels decay so rapidly, current procedures are wasteful of EPO, which is costly (current therapeutic protocols use in each injection about 70 times the amount of EPO naturally present in the whole of the patients body). Yet further, current protocols risk the serious side-effects mentioned above. These drawbacks are only exacerbated by the fact that subjects suffering from deficiencies treatable by EPO have to be treated over their entire lifetime.

Some attempts have been made to solve the problems caused by EPO's short *in vivo* half-life by presenting EPO in a sustained-release preparations. One such attempt is described in published PCT application WO92/04891 ("Chugai").

Chugai describes the microencapsulation of EPO into particles comprised of a biocompatible polymer (polylactide or polylactide coglycolide). Chugai's particles have an average

particle diameter of from 5 to 100 μm, a maximum particle diameter of 150 μm or less and a coefficient of variation of 50% or less (this is a measure of the degree of diversity in the size of the particles. It is defined as (standard deviation of size) ÷ (average particle diameter) x 100). Therefore, Chugai describes sustained-release preparations comprising particles having a particular range of sizes and a low degree of variation in size, i.e. uniformly-sized particles.

Within the range of sizes described by Chugai, preparations of particles having maximum particle sizes of from about 15μm to about 80μm are exemplified. Small particles having a maximum diameter of 15μm and an average diameter of 8μm are shown to release EPO at an approximately constant rate over a period of 1 month, whilst a preparation comprising larger particles, more heterogeneous in size and having a maximum diameter of about 80μm are unsatisfactory as they burst easily.

Therefore, known sustained-release EPO preparations comprising small particles are effective only for one month whilst preparations comprising larger particles are not stable enough to effect sustained release.

Surprisingly, the present inventors have found that an effective sustained-release preparation for the delivery of EPO can be formulated using preparations of polylactide coglycolide microspheres whose size distribution is bimodal, such that there are two peaks, an upper and a lower peak, in particle size. Surprisingly, the preparations of the invention have been found to release EPO heterogenously in time. After an initial rapid burst, EPO is released at a constant rate that suggests that they will be effective over as much as 1 year or longer. Thus, the preparations of the invention release EPO in two phases, a fast phase and a slow phase.

Some preparations of microspheres with heterogenoeous release characteristics have been obtained. For example, Yan <u>et al</u> (<u>Vaccine</u> vol. 13, No. 7 pp 645-651; 1995) describe a

preparation of homogeneously sized microparticles that release anti-ricin IgG in two bursts over time. However, the preparations of the invention comprise EPO, not anti-ricin IgG, and do not release EPO in bursts. Indeed, this would be undesirable, as it would lead to undesirable peaks in EPO levels in the patient.

The preparations of the invention allow long term maintenance of EPO at physiological levels in the bloodstream of the subject, thus eliminating the need for frequent administration of EPO and reducing the risk of thrombosis and hypertension. Thus they are clinically advantageous over preparations in which EPO is not associated with a sustained release mechanism and over known sustained-release preparations, which are not effective over long periods.

Accordingly, the invention provides:

a sustained release preparation comprising polylactide coglycolide (PLG) microspheres which comprise erythropoietin (EPO); said microspheres having a size distribution which is bimodal, having an upper peak and a lower peak in particle size;

a pharmaceutical composition comprising a preparation according to the invention and a pharmaceutically acceptable carrier; and

a water-in-oil-in-water emulsion process for producing a sustained release preparation comprising polylactide coglycolide (PLG) microspheres which comprise erythropoietin (EPO); said microspheres having a size distribution which is bimodal, having an upper peak and a lower peak in particle size.

Brief Description of the Drawings

Figure 1.

Microscopic analysis of the microspheres of the invention

A) Light microscopy of a typical batch of PLG microspheres loaded with EPO/BSA, (Magnification 100x).

B) Scanning electron microscopy of the same batch.

Figure 2.

Daily release of ^{125}I-EPO from PLG microsphere.
The released material is expressed as the logarithm of the percentage of the total encapsulated amount (A). Liberation of EPO is also shown as cumulative cpm released into the receiving fluid (B).

Figure 3.

Analysis in a 10% PAGE of ^{125}I-EPO released from PLG microspheres at different times after incubation at 37°C in PBS. After electrophoresis the gel was dried and exposed to X-ray film at -80°C A) after 1 day B) after 3 days C) after 5 + 6 days D) after 9 + 10 days E) after 14 + 15 days F) after 19 + 20 days G) after 22 + 23 days and F) after 26 + 27 days M) molecular weight marker (kDal).

Figure 4

Evolution of the haematocrit in 5/6 nephrectomised rats. The values shown are the average of 10 animals:

∇ Untreated control
○ Animals treated with a single dose (o) of rEPO at week 0.
▵ Animals treated with twice weekly doses (+) of rEPO.
● Animals treated with a single dose (*) of the EPO preparation of the invention at week 0.

Figure 5

Comparison between the sustained release preparation of the present ICGEB invention and that of Chugai (WO92/04891): comparison of the percentage of EPO released over the course of 1 month shows that the preparation of the invention releases EPO in a more sustained manner.

Figure 6

Depiction of the pKG5 plasmid vector used to express the EPO gene, which is inserted at the XhoI-Hind III sites of the vector.

Details of the pKG5 plasmid. Size: 5.8kb. Replicon: pMB1 (E.coli), SV40 (monkey COS). Selective phenotype for animal cells: G418. Selective phenotype for E.coli: Amp. Promoter: SV40. Cloning sites: HindIII, BamHI, Xhol.
The plasmid pKG5 comprises: a) the EcoRI-PvuII fragment of pBR322 (2394 bp) at positions 3693 to 176; b) the PvuII-HindIII fragment from SV40 (322 bp, 5191..5243, 1..270) at positions 176 to 498 containing the replication origin core region and the early mRNA promoter including the tandemly repeated 72bp segments; c) the BamHI-BcII fragment from SV40 (237 bp, 2533..2770) at positions 556 to 797, containing splice and polyadenylation signals from the SV40 early region; and d) a marker gene (neo), coding for the aminoglycoside 3' phosphotranferase, from transposon Tn5 confers resistance to the antibiotic G418. The neo gene (positions at pKG5: 1606 to 2400) is preceded by the promoter (fragment ClaI-BglII, positions at pkG5: 795 to 1571) and followed by the polyadenylation signal (SmaI-EcoRI fragment, positions at pKGF: 2571 to 3693) of the thymidine kinase (tk) gene from herpes simplex virus.

Figure 7

Bimodal distribution of the diameter of the microspheres of the invention. The microsophere diameter of the preparations obtained following the Eldridge et al protocol modified by a

variable stirring speed is shown. The bimodal distrubution of the microsphere diameter is clear.

The sustained release preparations of the invention comprise microspheres (also known as microcapsules). These are small particles prepared by encapsulating a pharmaceutically active agent, in this case EPO, in an outer layer consisting of the polymer polylactide coglycolide (PLG). Thus, the microspheres of the invention comprise a "core" of active material (EPO) surrounded by a layer of PLG.

These microspheres release the EPO at a rate determined by their size and chemical composition. In particular, the smaller the microspheres, the greater the rate of release. As the releasing mechanism is a combination of bulk and/or surface erosion and diffusion through the pores that exist in the polymer layer, it is possible to vary some factors (drug loading polymer molecular weight, composition, particle size and porosity) in order to vary the release characteristics of the EPO.

The preparations of the invention comprise microspheres of widely varying sizes, and these sizes are in a bimodal distribution. It is probable that this accounts for their ability to release EPO at relatively constant rates over long periods of time, with the larger microspheres releasing EPO slowly and the smaller ones doing so more rapidly.

In the preparations of the invention, the distribution of particle sizes is bimodal. This means that there are two peaks in particle size, an upper peak and a lower peak. These peaks are peaks in terms of the frequency of particles of a given size. Thus there are two sizes or size ranges in which high preparations of the total number of particles in the preparations are found.

Preferably, the upper peak occurs at from 30 to 150 μm, more preferably at from 50 to 130 μm, 50 to 100 μm or 100 to 130 μm. Most preferably, the upper peak occurs at from 50 to 70 μm.

The two peaks typically contain a high proportion of the total number of microspheres in the preparation. For example, each peak may contain up to 10, up to 20, up to 30 or up to 40% of the total number of microspheres. The two peaks may be of any size relative to one another. For example, the upper peaks may contain a higher proportion of the total number of microspheres that the lower one, or the lower peak may contain a higher proportion of the microspheres. Preferably, the upper peak contains a higher proportion of the microspheres than the lower one.

The width of each peak, and hence the proportion of the mcrospheres contained in it, can be determined by standard statistical techniques known in the art.

Preparations of the invention preferably have a minimum particle diameter of from 1 to 10μm, more preferably from 3 to 5μm; and a maximum particle diameter of greater than 150μm, preferably from 150 to 250μm, more preferably from 150 to 220μm and most preferably around 200μm, for example 180 to 190μm, 190 to 200μm, 200 to 210μm or 210 to 220 μm. As long as these conditions regarding maximum and minimum size are met, and as long as the composition has the desired release characteristics, there may be any proportion of the microspheres having any particular size.

The microspheres of the invention comprise, as an active ingredient, Erythropoietin (EPO). This EPO may be obtained from any suitable source. For example, it may be extracted from animals that produce it or it may be produced recombinantly. Recombinantly produced human EPO is preferred, and this is commercially available. Non-human EPO may also be used. Recombinantly produced EPO may be prepared by any suitable method, for example biosynthesis in transformed bacterial, e.g. *E. coli* cells, which generates unglycosylated EPO; biosynthesis in transformed mammalian, e.g. CHO or BHK, cells, which produces fully glycosylated EPO; or biosynthesis in baculovirus-infected insect cells. This does not have the characteristic mammalian glycosylation pattern essential for an

extended plasma half life. EPO from this source is extremely short lived although very active *in vivo*; for this reason, this baculovirus-derived EPO may be particularly suitable for delivery *in vivo* after entrapment in biodegradable microspheres according to the invention. It is envisaged that continuous EPO release according to the invention will compensate for the short half-life and give a good activity of clinical value.

Alternatively, commercially available EPO can be used. For reference, the industrial production of EPO is carried out on rollers at 37°C without CO_2. The culture is harvested every 48 hours for up to 10 days (5 harvests) before replacing it with fresh cells. For production, the media is DMEM plus antibiotic but lacking foetal calf serum, geneticin (G418) and methotrexate. The EPO obtained is of high purity with a specific activity of about 100000 u/mg. The yield of the process is about 50%.

Ultrapure recombinant human EPO suitable for use according to the invention is available from (Amersham) under Catalogue No. 286-EP as a sterile-filtered solution in 50% glycerol in 25 mM Hepes buffer, pH 7.2 and has a purity of >97%, as determined by SDS-PAGE visualized by silver stain, and a specific activity of greater than 100,00 units/A_{280}. The *in vitro* biological activity of this preparation, measured in a cell proliferation assay using a factor-dependent human erythroleukemic cell line, TF-1 (Kitamura, T. *et al.*, 1989, J. Cell. Physiol. **140**:323, gives an ED50 of 0.1 units/ml.

Also, tissue-culture grade EPO is available from (Amersham) under Catalogue No. 287-TC as a sterile-filtered solution of PBS with carrier, having a greater than 100,000 units/A_{280}. The *in vitro* biological activity of this preparation, measured in a cell proliferation assay using a factor-dependent human erythroleukemic cell line, TF-1 (Kitamura, T. *et al.*, 1989, J. Cell. Physiol. **140**:323) gives an EP50 of 0.1 units/ml.

EPO for use according to the invention amy be of any suitable purity. Preferably, it is substantially pure. EPO of at least

70%, at least 80%, at least 90%, at least 95%, or at least 99% purity is preferred.

For the purposes of the invention, the EPO may have any suitable specific activity. For example, EPO having an activity of from 50,000 to 150,000 μ/mg is preferred, with EPO having an activity of from 80,000 to 120,000 μ/mg, such as about 100,000 μ/mg being particularly preferred.

The microspheres of the invention may comprise any effective amount of EPO, and the amount of EPO contained in the microspheres will vary, for example with the size of the microspheres and with the therapeutic application. The microspheres of the invention comprise a polyactide coglycolide (PLG) capsule. PLG is a suitable material because it is biocompatible and biodegradable. This PLG may have any suitable characteristics. For example, it may have any average molecular mass, for example from 1000 to 20000 with PLG having an average molecular mass of 5000 to 10000 being preferred.

Similarly, the PLG may comprise any suitable proportions of lactide and glycolide moieties. For example, the ratio of lactide to glycolide may be from 90:10 to 10:90 with ratios of from 20:80 to 80:20 being preferred and ratios of from 40:60 to 60:40 being more preferred, and a ratio of 50:50 being most preferred. Lactide is optically active and any proportions of D and L isomers may be present, ranging from pure D-Lactide to pure L-lactide, with racemates comprising 50% D-lactide and 50% L-lactide being preferred.

The microspheres of the invention may be prepared by any means which results in a suitable distribution of particle sizes and other characteristics, as described herein. For example, EPO-containing microspheres may be prepared by a water-in-oil-in water emulsion process similar to that by the process of Eldridge et al, (1991: Infection and Immunity pp 2978-2986), but incorporating the modifications described in the Examples. The bimodal distribution of particle sizes according to the invention, which is atypical and surprising, is different from

that obtained by Eldridge et al. Typically, water-in-oil-in-water emulsion processes of the invention involve two emulsification steps, and this distribution is believed to be, at least to some extent, a function of the stirring speed profile during the formation of the second emulsion. (Mixing of PLG-Cl_2-CH_2 solution with EPO in polyvinyl alcohol). Unusually, this stirring was not performed at constant speed but rather at two speeds, 200 rpm for the first half of the process and 500 rpm for the second half. This bimodal profile is believed to be responsible for the bimodal EPO release profile of the microspheres of the invention.

Accordingly, preparative processes in which this second emulsification step takes place under a stirring speed regime are preferred. In a bimodal regime, it is preferred that the stirring speed during the first period is higher than that during the second.

Any suitable speed may be used in either period of stirring. For example, suitable speeds for the first period may be from 500 to 10000 rpm, preferably from 1000 to 10000 rpm, more preferably from 1500 to 2500 rpm or from 1800 to 2200 rpm. A particularly preferred speed for the first period is 2000 rpm. For the second period, suitable speeds may be from 100 to 5000 rpm, preferably from 100 to 1000 rpm, more preferably from 200 to 800 rpm or from 400 to 600 rpm. A particularly preferred speed is 500 rpm. In a bimodal regime the two agitation periods at different speeds may be of any suitable length, and of any suitable length in relation to each other. For example, the two periods may be equal or unequal in length. For example, the two periods may occupy, respectively, from 90% and 10% to 10% and 90% of the total emulsification period. It is preferred that the two periods occupy from 80% and 20 to 20% and 80% of the emulsification period, preferably from 60% and 40% to 40% and 60% of the period. In a preferred embodiment, the two periods occupy 50%, or around 50% each of the total emulsification period. The transition between the two different stirring speeds may take place at any speed, e.g. gradually or instantaneously.

It will be appreciated by those of skill in the art that preparation procedures will vary slightly depending on the nature of the microspheres to be produced; for example, preparative procedures may be tailored to give microspheres with a particular size distribution, or maximum or minimum particle size (diameter) for which they are intended.

The invention provides microsphere preparations obtained, or obtainable, by the processes of the invention.

The "core load" of EPO in the microspheres may vary between preparations or within a single preparation. For example, the core load of EPO may be from 0.1 to 10% of the total mass of the microspheres, with core loads of 0.5 to 5% being preferred and EPO core loads of 1 to 2% being particularly preferred.

Other proteins may also be incorporated into the microspheres, for example other pharmaceutically active proteins or pharmaceutically inactive proteins. This increases the total core load of protein and total core loads of 0.1 to 20% are preferred with total core loads of 7 to 10% being particularly preferred. One preferred inactive protein is human serum albumin (HSA), though others may be used as well or instead.

The sustained-release preparations of the invention typically have certain characteristics as far as their release of EPO *in vivo* or *in vitro* is concerned. Typically they release EPO at an approximately constant rate over a long period, typically greater than one month, and preferably for 1 to 6 months, from 6 months to one year or more.

Prior to this constant-release phase, there is typically an initial burst of rapid EPO release, wherein a substantial proportion of the total EPO may be released, for example, up to 40% of the total encapsulated EPO, such as up to 10% or up to 20 or 30%. Thus, the microsphere preparations of the invention release EPO in a biphasic manner. It is believed that this is, at least partly, caused by the bimodal size distribution of the particles, and that this, in turn, is at least partly caused by

the bimodal stirring regime which may be used in preparing the microspheres.

The microspheres of the invention may be administered in any suitable amount and by any suitable route in order to deliver EPO to human or animal, preferably human, subjects.

Typically, each dose of the preparation will contain from 1000 to 100000 units of EPO, preferably 5000 to 50000 units, more preferably 10000 to 20000 or 20000 to 30000 units, for an adult human of average size. For larger or smaller humans, or for animals, these doses can be adjusted in relation to body mass. For example, a typical dose for research uses in mice may comprise from 10 to 100 units of EPO, preferably from 20 to 50 units of EPO, for example 20 to 25 units.

Typically, the preparations of the invention are administered to subjects deficient in EPO and the dose size and release characteristics of the preparation will be adjusted to increase the level of EPO in the subject's bloodstream towards the level found in normal subjects. Preferably, the release characteristics of the preparation will be such that a normal or near-normal level is maintained over a long period of time, 95 mentioned above.

Any suitable dosage regime that maintains a suitable blood level of EPO may be used. For example, a dose may be administered from a 1 to 50 times per year, within monthly and bimonthly dosages being preferred.

The sustained release preparations of the invention may be administered by any suitable route, with injection being preferred, parenteral injection being particularly preferred and subcutaneous injection being most particularly preferred.

The invention is now illustrated by means of the following Examples and Comparative Example.

<u>EXAMPLES</u>

Example 1

Development of a cell line producing human erythropoietin

The erythropoietin (EPO) gene was isolated from human genomic DNA using standard PCR and cloning techniques.

The EPO gene (approx 2.1 kb) was isolated from genomic DNA prepared from the white blood cells of healthy individuals. PCR techniques using primers designed in the fashion shown in the enclosed sequence.

The cloning and sequencing manipulations were done following standard genetic engineering methods (Sambrook *et al*, 1987. Molecular Cloning: a laboratory manual ed. C.S.H. University Cold Spring Harbor). The gene sequence was modified without changing the amino acid sequence to allow easier genetic manipulation and. The amino acid and the nucleotide sequence were checked in the final construct. The gene was cloned into expression vector pKG5 (that confers geneticin (G418) resistance) and was introduced into CHO DHFR$^-$ cells together with the plasmid pSV2 DHFR (that complements the DHFR (dihydrofolate reductase) insufficiency.

The vector map of the pKG5 plasmid is given in Fig. 6, this vector having been constructed over 12 years ago using fragments from known vectors.

The SV40 expression unit segment was derived from the plasmid pSV1 described by Krieg et al., J. Mol. Biol. 180, 615-643 (1984). It was modified for the cloning site using synthetic oligonucleotides.

The TK neo gene has the promoter and poly A sites of Herpes TK (Colbere-Gerapin et al., J. Molec. Biol. 150 1-14 (1981).

The EPO sequence inserted includes 2 nucleotides preceding the ATG and 15 nucleotides following the stop codon.

The cells were selected for G418 resistance and checked for EPO production. The EPO producing clones were subjected to gene amplification by selection with methotrexate (Mtx).

The end product was a cell line that produces 10^7 units of EPO/litre of culture per 24 hours. The cells can be grown in αMEM medium lacking ribo-and deoxyribonucleotides (GIBCO 072-02000) complemented with gentamicin 50 mg/l, L-glutamine 2 mM, geneticin (G418) 500 mg/l, Fetal Calf Serum (FCS) (dialysed) 10%, Methotrexate 10^{-7}M.

The mRNA and amino acid sequences of this EPO were compared to these of the industrial product.

<u>Sequence determination</u>

Amino acid sequences were determined on the purified EPO using a model 477A protein microsequencer from Applied Biosystems. According to the method of Hewick *et al*. (J. Biol. Chem., 256, 7990-7997, 1981).

The mRNA sequences were determined by cDNA amplification with the specific primers from total RNA prepared from the producer cell line. The techniques for amplification and sequence were the standard ones (Maniatis, Fritsch and Sambrook, 1982. Molecular cloning: a laboratory manual ed. C.S.H. University Cold Spring Harbor).

For reference, units of EPO are measured according to the International Convention for doing so, with EPO activity calibrated against the second international reference preparation of erythropoietin (Annable, L. *et al*., 1972, Bull. Wld. Hlth. Org **47**:99)using an *in vivo* bioassay which measures the incorporation of ^{56}Fe into red blood cells of exhypoxic polycythemia mice (Cotes and Bangham, 1961, Nature **191**:1065). Broadly speaking, 5mg of purified EPO corresponds to 10^6 international units.

Example 2

Production of EPO in a baculovirus/insect cell system

EPO cDNA was prepared from RNA extracted from the cell line described in Example 1. The cDNA was placed under the control of the polyhdrin promoter (Quelle *et al* (1992) Protein Expression and purification 3, pp461-469) and a recombinant baculovirus system producing EPO was constructed.

Example 3

Encapsulation of human EPO in biodegradable-biocompatible polymeric microspheres.

Human ^{125}I labelled EPO (Amersham) was entrapped in PLG, in which the ratio between the glycolide and lactide monomers was 50:50, according to the method of Eldridge *et al* (1991: Infection and Immunity pp2978-2986), with certain modifications. This polymer is hydrolysed much faster than those having a higher proportion of either monomer. The procedure used was that of an emulsion process (water-in-oil-in-water emulsion) as described by (Eldridge et al), with certain modifications as described below. The particle size distribution was from 3-5μm to 200μm in diameter. The preparation was then freeze-dried and maintained in a dry state until use. The amount of protein in the microparticles was determined by a bicinchoninic acid protein assay (Pierce Chemical Co, Rockford, Illinois) after disruption of the microparticles and extraction of the entrapped protein, with the microparticles being disrupted and the protein extracted as follows: 20-25 mg of microspheres were dissolved in 2 ml of Cl_2CH_2 (methylene chloride) and the protein extracted 3 times with 0.5 ml of 0.1N NaOH. The aqueous layer was subjected to protein determination by the bichinconic assay method (Pierce, see above).

The emulsification technique of Eldridge <u>et</u> <u>al</u> was modified to obtain a bimodal distribution of the microspheres' diameter (see Fig. 7). In the second emulsification step (Eldridge <u>et</u> <u>al</u>) the stirring speed was initially 2000 rpm and half way through the process it was switched to 500 rpm.

The amount of protein found to be present in the microspheres (the "core load") is a function of the protein concentration to be entrapped in the aqueous solution used for the first emulsion. 12,000 units of recombinant EPO and 15 mg of human serum albumin were dissolved and entrapped in 1 g of Polylactide coglycolide (PLG). The protein entrapment is 50% efficient and the PLG recovery 80% efficient. Hence the core loading was about 1%. Earlier studies on the release of HSA from PLG microspheres (Bio/Technology 1990, 8, 755-758) have indicated that relatively high protein content (11.6% HSA/PLG w/w) was required to obtain continuous release of HSA in buffers containing physiological concentration of salt. As the amount of EPO needed for the present invention is very small HSA (Human Serum Albumin) was added as a carrier to obtain a total protein load of about 7-10% W/W PLG.

Around 50% of the protein was entrapped by this process. Figure 1 shows the appearance of this material analysed by (part a) light microscopy and by (part b) scanning electron microscopy.

Toxicity

Preliminary toxicity tests can be carried out on the EPO preparation of the invention following International standard pharmacopoeia procedures.

(a) tests for acute toxicity in mouse and guinea pig via both venous or subcutaneous administration, With a view to determining the maximum tolerated and maximum repeatable doses.

(b) Toxicity tests on repeated doses (one to three months), administering the EPO for mutation of the invention subcutaneously.

(c) Reproductive toxicity tests carried out on animals treated in (b).

All of a) to c) revealed extremely low toxicity of the EPO preparation of the invention even lower than EPO itself.

Also,

(d) Ames tests for mutagenesis can be performed on the final preparation (Ames, B.N., McCann J. and Yamasaki E., Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test, *Mutat. Res.*, **31**, 347-363, 1975). These also revealed low toxicity.

The EPO preparation of the invention was tested for toxicity, *in vitro* and *in vivo* activity.

Example 4
***In vitro* studies**

The *in vitro* release of EPO from PLG microspheres was examined by incubation of 10 mg of microspheres in 1 ml of receiving fluid. (Phosphate buffered saline-sodium azide 0.02% maintained at 37°C withdrawn periodically and replaced with the same amount of fresh fluid). As can be seen in Figure 2, approximately 35% of the encapsulated EPO is available for quick release during the first and second days followed by a slower release for a period of 30 days. The amount of EPO released per day from the PLG after the initial burst varies between 0.5 and 1%, based on the total amount initially present.

Overall, a release profile having the following phases was observed (Fig 2).

The first phase is characterised by an initial burst of EPO release (rapid release from surface bound and incompletely encapsulated protein). In the second phase the release occurs by diffusion through aqueous pores and channels as the molecular weight of the polymer decreases.

The integrity of this protein, after being kept at room temperature for long periods of time, was checked by polyacrylamide gel electrophoresis (PAGE). As can be seen in Figure 3, the gel migration of the material taken at different times during the course of the experiment corresponds to the known molecular weight of EPO (34kDa) and has the electrophoretic mobility of the original material taken before encapsulation. However, since the molecular integrity as measured in a PAGE is not enough to assure physiological activity, the *in vivo* effect of the release material was also evaluated.

PLG-EPO microspheres were incubated for 30 days at 37°C in PBS. Subsequently, an aliquot of the receiving fluid was injected in posthypoxic polycytemic mice, a standard EPO measurement (Cotes and Bangham (1961): Nature 191 pp1065-1067; Cotes (1988): British Medical Journal pp296-806). The released EPO stimulated the uptake of ^{59}Fe in mice, indicating that its physiological activity remained unaltered after the whole procedure.

Example 5

***In vivo* studies**

(a) <u>Kinetics of Erythropoietin release *in vivo* from the PLG microspheres</u>.

A group of fifteen mice were subcutaneously injected for *in vivo* studies with the same batch of ^{125}I-EPO. Animals were sacrificed at different times (1 to 21 days) after injection. The radioactivity was measured in samples collected from liver, thyroid gland, blood and urine. From these data it can be seen that, as in the case of the *in vitro* release, there is an initial burst followed by a steady release at least until day 21 when the last animal was sacrificed.

(b) Physiological activity *in vivo* in polycytemic posthypoxic mice.

Physiological activity was measured in posthypoxic polycytemic mice as described in (a) above using EPO produced by the cell

line described in Example 1. This method is limited by its short duration as the production of endogenous EPO by posthypoxic mice restarts after a few days. Nevertheless a definitive prolonged effect was observed for up to 5 days after injection of microencapsulated EPO.

The entrapment of EPO in microspheres of the invention allows a biologically active form to be kept for long periods at room temperature avoiding stabilizers and/or cold chain.

(c) Biological activity *in vivo* in the 5/6 nephrectomised rat model.

To measure the sustained biological activity of EPO retard *in vivo* we have used the well proven rat model of anaemia lessening. Rats were subjected to 5/6 nephrectomy (Waynforth *et al* (1992) "Experimental and Surgical Techniques in the Rat"; Academic Press) this produces a mild anaemia giving a 42% Haematocrit (Hct). This anaemia can be corrected to a Hct of 50% by treatment with recombinant EPO, 25 units intraperitioneal, twice weekly (Garcia, *et al* (1988): PNAS 85 pp6142-6146). Fig. 4 shows the values of the Hct measured in tail blood of conscious rats after renal ablation without treatment (inverted triangles ▼), treated with a single dose of rEPO (open circles 0), twice weekly does of rEPO for 4 weeks open triangles or with the EPO preparation of the invention (full circles ●). We have repeated these experiments using a single injection of EPO retard and following up the evolution of the Hct for 4 weeks.

It can be seen that a single injection of the formulation of the invention has the same biological effect as 8 injections of rEPO. Considering the characteristically short life *in vivo* of unencapsulated EPO and that several doses are necessary to maintain a physiological level of it , it is easy to see that the clinal benefits of using the EPO formulation of the invention to treat human patients.

Comparative Example 1

Comparison between the sustained release preparation of Chugai (Example 1 in WO 92/04891) and that of the present invention (as prepared in Example 3).

Figure 5 compares the *in vitro* release characteristics of the two preparations, in terms of the percentage of EPO remaining in the microspheres at a series of time-points (as a fraction of the total EPO originally encapsulated). Clearly, Chugai's preparation releases EPO at an approximately constant rate over the course of one month, whereas the EPO preparation of the present invention, after an initial rapid burst, releases a far smaller fraction of its encapsulated EPO (approximately 40%) in the same time. After the initial burst, the rate of release is approximately constant at around 3% per week. At this rate of release, EPO could be maintained of physiological concentrations for several months, or even as much as one year or more. Thus, it can be seen that the preparation of the invention has very different release characteristics than those known in the art, as represented by the Chugai preparation. Accordingly, it is clear that the preparation of the invention will provide a sustained release of EPO over a far longer period than previously known preparations, as represented by that of Chugai.

CLAIMS

1. A sustained release preparation comprising polylactide coglycolide (PLG) microspheres which comprise erythropoietin (EPO); said microspheres having a size distribution which is bimodal, having an upper peak and a lower peak in particle size.

2. A preparation according to claim 2 wherein the minimum diameter of the microspheres is from 1 to 10 μm and the maximum diameter is greater than 150 μm.

3. A preparation according to claim 1 or 2 wherein the minimum diameter of the microspheres is from 3 to 5 μm.

4. A preparation according to any one of the preceding claims wherein the lower peak in particle size is at up to 20 μm.

5. A preparation according to claim 4 wherein the lower peak is at up to 10 μm.

6. A preparation according to claim 5 wherein the lower peak is at up to 5 μm.

7. A preparation according to any one of the preceding claims wherein the upper peak in particle size is at from 30 to 150 μm.

8. A preparation according to claim 7 wherein the upper peak is at from 50 to 130 μm.

9. A preparation according to claim 8 wherein the upper peak is at 50 to 100 μm.

10. A preparation according to claim 8 wherein the upper peak is at from 100 to 130 μm.

11. A preparation according to any one of the preceding claims wherein, in the PLG, the ratio of lactide to glycolide moieties is from 60:40 to 40:60.

12. A preparation according to claim 11 wherein the ratio is 50:50.

13. A preparation according to any one of the preceding claims wherein the EPO is produced by expression of nucleic acid encoding EPO in bacterial cells or in a baculovirus/insect cell system.

14. A preparation according to any one of the preceding claims wherein EPO accounts for from 0.1 to 10% of the weight of the microspheres.

15. A preparation according to any of the preceding claims wherein the PLG has an average molecular mass of from 1,000 to 20,000.

16. A preparation according to any one of the preceding claims wherein the EPO has a specific activity of from 50,000 to 150,000 u/mg.

17. A preparation according to any one of the preceding claims wherein the microspheres further comprise at least one second protein in addition to EPO.

18. A preparation according to claim 17 wherein the second protein accounts for from 0.1 to 20% of the weight of the microspheres.

19. A preparation according to claim 17 or 18 wherein the second protein is a pharmaceutically inactive carrier protein.

20. A preparation according to any one of claims 17 to 19 wherein the second protein is human serum albumin.

21. A preparation according to any one of the preceding claims for use in a method of treatment of the human or animal body by therapy.

22. Use of a preparation according to any one of the preceding claims in the manufacture of a medicament for stimulating the proliferation and/or differentiation of erythroid cells, or for increasing the haematocrit and/or haemoglobin concentration of blood.

23. A pharmaceutical composition comprising a preparation according to any one of the preceding claims and a pharmaceutically acceptable carrier.

24. A water-in-oil-in-water emulsion process for producing a sustained release preparation comprising polylactide coglycolide (PLG) microspheres which comprise erythropoietin (EPO); said microspheres having a size distribution which is bimodal, having an upper peak and a lower peak in particle size.

25. A process according to claim 24 wherein, during the second emulsification step, the mixture is stirred at two different speeds.

26. A process according to claim 25 wherein the first stirring speed is higher than the second stirring speed.

27. A process according to claim 25 or 26 wherein the first stirring speed is from 1500 to 2500 rpm.

28. A process according to any one of claims 25 to 27 wherein the second stirring speed is from 100 to 1000 rpm.

29. A process according to any one of claims 25 to 28 wherein the period of stirring at the first speed occupies from 40% to 60% of the period of stirring during the second emulsification step.

30. A process according to any one of claims 25 to 30 which is a process for producing a preparation according to any one of claims 1 to 22.

31. A sustained release preparation obtainable by a process according to any one of claims 21 to 29.

The Patent Office

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Application No:	GB 9617732.4	**Examiner:**	Mr S J Pilling
Claims searched:	1 to 31	**Date of search:**	7 November 1996

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Search Report under Section 17

Databases searched:

UK Patent Office collections, including GB, EP, WO & US patent specifications, in:

UK Cl (Ed.O): A5B (BLM, BNC, BHA, BJB, BJC)

Int Cl (Ed.6): A61K 9/16, 9/50, 38/18

Other: ONLINE: CAS ONLINE, WPI, JAPIO, CLAIMS, DIALOG/MEDICINE, DIALOG/PHARM

Documents considered to be relevant:

Category	Identity of document and relevant passage	Relevant to claims
A	WO 93/25221 A1 (ALKERMES) see page 3 lines 13 to 24, page 5 lines 10 to 17 and Example 4.	-
A	WPI Abstract Accession No. 92-131866/16 & WO 92/04891 A1 (CHUGAI) 02.04.92 (see abstract).	-

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
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